Antonie van Leeuwenhoek (2014) 106:543–553
DOI 10.1007/s10482-014-0226-0

ORIGINAL PAPER

Nocardia vulneris sp. nov., isolated from wounds of human patients in North America

Brent A. Lasker · Melissa Bell · Hans-Peter Klenk · Cathrin Spröer · Peter Schumann · June M. Brown

Received: 7 March 2014 / Accepted: 25 June 2014 / Published online: 12 July 2014

Abstract *Nocardia* species are ubiquitous in the environment with an increasing number of species isolated from clinical sources. From 2005 to 2009, eight isolates (W9042, W9247, W9290, W9319, W9846, W9851^{T}, W9865, and W9908) were obtained from eight patients from three states in the United States and Canada; all were from males ranging in age from 47 to 81 years old; and all were obtained from finger ($n = 5$) or leg ($n = 3$) wounds. Isolates were characterized by polyphasic analysis using molecular, phenotypic, morphologic and chemotaxonomic methods. Sequence analysis of 16S rRNA gene sequences showed the eight isolates are 100 % identical to each other and belong in the genus *Nocardia*. The nearest phylogenetically related neighbours were found to be the type strains for *Nocardia altamirensis* (99.33 % sequence similarity), *Nocardia brasiliensis* (99.37 %), *Nocardia iowensis* (98.95 %) and *Nocardia tenerifensis* (98.44 %). The G+C content of isolate W9851^{T} was determined to be 68.4 mol %. The DNA–DNA relatedness between strain W9851^{T} and the *N. brasiliensis* type strain was 72.8 % and 65.8 % when measured in the laboratory and in silico from genome sequences, respectively, and 95.6 % ANI. Whole-cell peptidoglycan was found to contain *meso*-diaminopimelic acid; MK-8-$(H_4)_{\omega\text{-cyc}}$ was identified as the major menaquinone; the major fatty acids were identified as $C_{16:0}$, 10 Me $C_{18:0}$, and $C_{18:1\ w9c}$, the predominant phospholipids were found to include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides; whole-cell sugars detected were arabinose and galactose; and mycolic acids ranging from 38 to 60 carbon atoms were found to be present. These chemotaxonomic analyses are consistent with assignment of the isolates to the genus *Nocardia*. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra of the clinical isolates showed genus and species level profiles that were different from other *Nocardia* species. All isolates were resistant to ciprofloxacin, clarithromycin and imipenem but were susceptible to amikacin, amoxicillin/clavulanate, linezolid and trimethoprim/sulfamethoxazole. The results of our polyphasic analysis suggest the new isolates obtained from wound infections represent a novel species within the genus *Nocardia*, for which the name *Nocardia vulneris* sp. nov. is proposed, with strain W9851^{T}

Electronic supplementary material The online version of this article (doi:10.1007/s10482-014-0226-0) contains supplementary material, which is available to authorized users.

B. A. Lasker (✉) · M. Bell · J. M. Brown
Bacterial Special Pathogens Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Disease, Centers for Disease Control and Prevention, Building 17, Room 2025, Mailstop G-11, 1600 Clifton Road, Atlanta, GA 30333, USA
e-mail: blasker@cdc.gov

H.-P. Klenk · C. Spröer · P. Schumann
Leibniz-Institute DSMZ - German Collection of Microorganisms and Cell Cultures, 38124 Brunswick, Germany

(= DSM 45737^T = CCUG 62683^T = NBRC 108936^T) as the type strain.

Keywords *Nocardia vulneris* · Polyphasic analysis · Wet-lab · In-silico DDHs · Actinomycetes · Wound infection

Introduction

The genus *Nocardia*, established by Trevisan (1889) is comprised of Gram-stain positive, weakly acid-fast, aerobic, nonmotile, saprophytic bacteria. At present, there are 87 *Nocardia* species with validly published names *List of Prokaryotic Names with Standing in Nomenclature* (http://www.bacterio.net/nocardia/). While the majority live in the soil or water as saprophytes, over 30 species have been reported to be responsible for human infections. The major clinical manifestations for nocardiosis include the pulmonary and central nervous system, systemic or disseminated, and cutaneous infections. Cutaneous nocardiosis is believed be the result of primary infection due to trauma or puncture, or from a disseminated infection (Corti and Fioti 2003; Yamaguchi et al. 2013). Following a retrospective analysis of 16S rRNA gene sequence data obtained from clinical isolates submitted to the Special Bacteriology Reference Laboratory (SBRL) for identification from 2005 to 2009, we identified eight clinical isolates that suggested a novel pathogenic *Nocardia* species. The purpose of our investigation was to clarify the taxonomic position of these clinical isolates using molecular, phenotypic, morphologic and chemotaxonomic analysis. Analysis was consistent with the conclusion that the eight clinical isolates are members of a new species of the genus *Nocardia* for which *Nocardia vulneris* is the proposed name with isolate W9851^T (= DSM45737^T = CCUG 62683^T = NBRC 108936^T) being the type strain.

Materials and methods

Bacterial strains and culture

Between 2005 and 2009, eight clinical isolates, W8477, W9247, W9290, W9319, W9846, W9851^T, W9865 and W9908 were obtained from wound infections from patients in three states in the United States and Canada. All isolates were sent to the SBRL at the Centers for Disease Control and Prevention for identification; five isolates, (W9247, W9290, W9319, W9865 and W9908) were obtained from finger wounds, and three isolates (W8477, W9846, and W9851^T) were obtained from leg wounds. Following 16S rRNA gene analysis, all isolates were identified to belong to the genus *Nocardia*.

To examine morphological features, isolates were grown aerobically using trypticase soy broth (TSB), heart infusion agar (HIA; Becton, Dickinson and Co, Sparks, MD) supplemented with 5 % rabbit blood, trypticase soy agar (TSA) supplemented with 5 % sheep blood (Becton, Dickinson and Co, Sparks, MD), heart infusion agar, and Middlebrook and Cohn 7H11 agar with Oleic Albumin Dextrose Catalase supplement (OADC) at 35 °C for 4–7 days and then examined for microscopic and macroscopic features.

Nocardia altamirensis DSM 44997^T and *Nocardia tenerifensis* DSM 44704^T were obtained from the Deutsche Sammlung von Mikrooganismen und Zellkulturen (DSMZ). *Nocardia brasiliensis* NBRC 14402^T was obtained from the NITE Biological Resource Center. *N. brasiliensis* HUJEG-1 (= ATCC 700358 was obtained from the American Type Culture Collection (ATCC). *Nocardia iowensis* NRRL 5646^T was provided by the Agricultural Research Service Culture Collection (NRRL).

Genotypic analysis

16S rRNA gene sequence analysis

Purification of genomic DNA, amplification of a 1,441-bp 16S rRNA gene fragment, primers and DNA sequencing were previously described (Lasker et al. 2011). DNA sequences were compared to GenBank reference sequences using BLASTn software. Sequences were aligned using CLUSTAL W found within the MEGA5.0 software (Tamura et al. 2011). Phylogenetic analysis and corresponding trees were constructed using the algorithms for neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum parsimony (Kluge and Farris 1969) using MEGA5.0 software (Tamura et al. 2011). The neighbour-joining tree in Fig. 1 shows the relationship of the eight clinical isolates within the

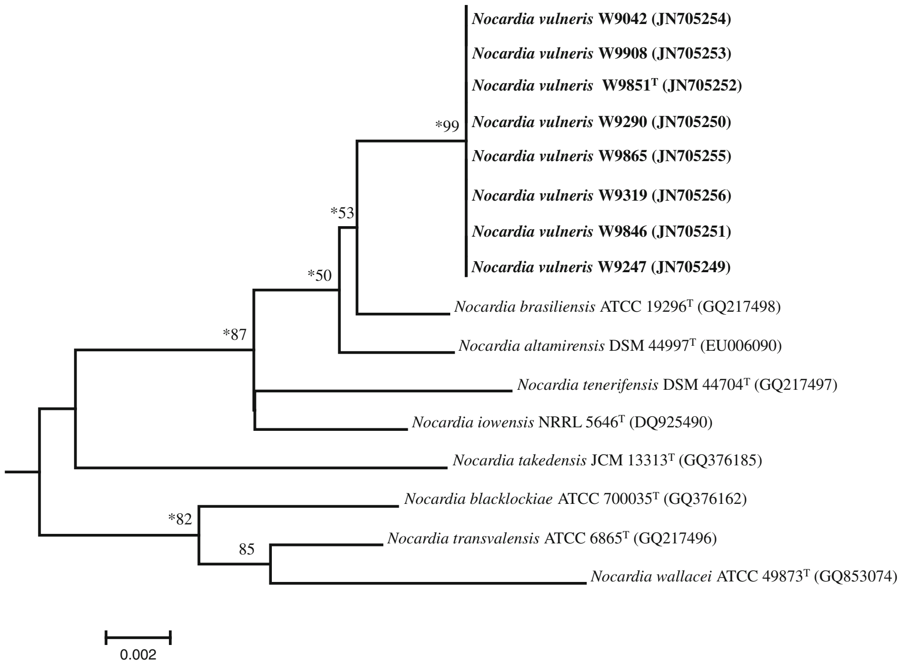

Fig. 1 Neighbour-joining phylogenetic tree, within the *Nocardia* genus, based on 1,441-bp 16S rRNA gene sequence for strain W9851^T. Bootstrap percentages based on 1,000 replicates; only values ≥50 % are shown. *Bar*, 0.005 substitutions per nucleotide position. Branches also obtained using maximum-likelihood and maximum-parsimony algorithms are indicated using asterisks. The extended tree from which this figure was taken is available as supplementary Fig. S1

Nocardia genus. Bootstrap replications were based on 1,000 replicates; only bootstrap values ≥50 are shown.

DNA–DNA hybridization

For laboratory determination Cells were disrupted using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and DNA in the crude lysates was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out in duplicate as described by De Ley et al. (1970) under consideration of the modification by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostat 6 × 6 multicell changer and a temperature controller with in situ temperature control. Intraspecies DNA–DNA hybridization studies between the clinical isolates were omitted due to the low probability inferred from 16S rRNA gene similarities (100 %) that these strains would not belong to the identical species (Meier-Kolthoff et al. 2013b). The method of Mesbah et al. (1989) was performed to determine the G+C content of the novel type strain and later confirmed from the draft genome sequence.

For in silico determination. Cells were freeze-dried and DNA extracted with standard procedures at LGC Genomics, Berlin. A paired-end DNA library was constructed and sequenced in one-half channel on an Illumina MiSeq V2 (2 × 250 bp mode). Error corrected sequence reads were *de novo* assembled with Newbler. In-silico hybridization with the 129 contig draft genome sequence of *N. brasiliensis* NBRC 14402^T (BAFT00000000) was performed using the Genome-to-Genome Distance Calculator (GGDC) 2.0 (Meier-Kolthoff et al. 2013a) through the DSMZ web service (ggdc.dsmz.de) and the ANI calculator (Goris et al. 2007).

Phenotypic analyses

Decomposition tests for adenine, casein, esculin, hypoxanthine, tyrosine, urea and xanthine; utilization of 22 carbohydrates as sole source of carbon; utilization of acetamide and citrate; arylsulfatase production and nitrate reduction; growth in lysozyme, growth at

25, 35 and 45 °C; Gram and modified Kinyoun acid-fast staining, were all conducted as previously described (Berd 1973; Conville and Witebsky 2007; Conville et al. 2008; Weyant et al. 1996; Yassin et al. 1995).

MICs to 11 antimicrobial agents were determined following the guidelines and interpretative breakpoints as recommended for the genus *Nocardia* by the CLSI (CLSI 2011) for ampicillin, amikacin, amoxicillin/clavulanate, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, moxifloxacin and trimethoprim/sulfamethoxazole.

Chemotaxonomic analyses

Assays of diaminopimelic acid stereoisomers and whole-cell sugars were performed by thin-layer chromatography using the methods described previously (Rhuland et al. 1955; Lechevalier and Lechevalier 1970). Isoprenoid quinones and polar lipids were extracted, purified and analyzed by the methods described by Minnikin et al. (1984). Analysis of isoprenoid quinones by HPLC was performed as described by Kroppenstedt (1982, 1985). Cellular fatty acids were prepared by the method of Klatte et al. (1994) and the fatty acid methyl esters were then separated as described by Sasser (1990) using the Microbial Identification System (MIDI, Inc., Sherlock version 6.1). Standardization of the physiological age of the clinical isolates and reference strains cultures was obtained by choosing the sector from a quadrant streak of culture plates. Mycolic acid analysis was conducted as described (Kroppenstedt 1982, 1985; Baba et al. 1997). Mass spectra were recorded and analyzed using a Microflex L20 MALDI-TOF mass spectrometer and MALDI Biotyper 3.1 software (both Bruker Daltonics) as described previously (Töth et al. 2008).

Results

Molecular analysis

All eight 16S rRNA gene sequences obtained from the novel clinical isolates were found to be 100 % identical to each other (GenBank accession numbers JN705249 to JN705256). BLASTn software (https://www.ncbi.nlm.nih.gov/blast/ was used to submit consensus 16S rRNA gene sequences for comparison of sequence similarity in the GenBank database. The eight clinical isolates formed a highly supported cluster within the genus *Nocardia* (Supplementary Fig. 1). Type strains showing the highest sequence similarity with the novel clinical isolates in the genus *Nocardia* were *N. altamirensis* DSM 44997^T (99.33 % similarity), *N. brasiliensis* ATCC 19296^T (= NBRC 14402^T; 99.37 %), *N. iowensis* NRRL 5646^T (98.95 %) and *N. tenerifensis* DSM 44704^T (98.44 %). Differences in sequence similarity from the novel clinical isolates to *Nocardia* type strains ranged from 9 bp for both *N. altamirensis* and *N. brasiliensis* to 21 bp for *N. tenerifensis*. Figure 1 shows a neighbour-joining tree showing the relationship to the closest related species of the genus *Nocardia*.

Laboratory DNA–DNA hybridization studies were performed in duplicate between the clinical isolate W9851^T, *N. altamirensis* DSM 44997^T, *N. brasiliensis* NBRC 14402^T and *N. brasiliensis* HUJEG-1 (= ATCC 700358). The level of DNA–DNA relatedness between W9851^T and strains *N. altamirensis* DSM 44997^T and *N. brasiliensis* NBRC 14402^T was 13.8 ± 2.5 and 72.5 ± 5.0 %, respectively. The borderline value for species delimitation between strain W9851^T and the type strain of *N. brasiliensis* was also confirmed by an in silico DDH of the W9851^T draft genome sequence (9.3 Mbp, 136 contigs; GenBank accession number JNFP00000000) and the *N. brasiliensis* NBRC 14402^T draft genome sequence (8.9 Mbp, 129 contigs, [BATF00000000]), which gave a value of 65.8 ± 2.9 % DDH. Comparison with the genome sequence of a second (non-type) strain of *N. brasiliensis* HUJEG -1 (= ATCC700358, 9.4 Mbp complete genome [CP003876]) gave a digital DHH value of 61.2 ± 2.8 %. Comparison of the draft genome sequences of strain W9851^T and *N. brasiliensis* NBRC 14402^T using the ANI calculator resulted in an ANI value of 95.6 %. The G+C content for strain W9851^T was determined to be 68.4 mol % (wet-laboratory procedure) and 68.1 mol % and is consistent with the range for members of the genus *Nocardia*.

Phenotypic characters

All clinical isolates were found to be aerobic, Gram-stain positive, non-motile, filamentous, and weakly acid-fast with the modified Kinyoun acid-fast stain

Table 1 Phenotypic properties of eight *N. vulneris* clinical isolates

Characteristics	W9042	W9247	W9290	W9319	W9846	W9851	W9865	W9908
Utilization of:								
Adonitol	−	−	−	−	−	−	−	−
L-arabinose	−	−	−	−	−	−	−	−
D-cellobiose	−	−	−	+	−	−	−	−
Dulcitol	−	−	−	−	−	−	−	−
i-Erythritol	−	−	−	−	−	−	−	−
D-fructose	+	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+
i-myo-inositol	+	+	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	−	−
D-mannitol	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+w
Melibiose	−	−	−	−	−	−	−	−
Raffinose	−	−	+w	+w	−	−	−	−
L-rhamnose	−	−	−	−	−	−	−	−
Salicin	+	+	+w	+w	+	+	+	+
D-sorbitol	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−
Trehalose	+	+	+	+	+	+	+	+
D-xylose	−	−	−	−	−	−	−	−
Growth at 25 °C	+	+	+	+	+	+	+	+
Growth at 35 °C	+	+	+	+	+	+	+	+
Growth at 45 °C	−	−	−	−	−	−	−	−
Arylsulfatase production	−	−	−	−	−	−	−	−
Hydrolysis of:								
Adenine (21 days)	+	+	+	+	+	+	+	+w
Casein (14 days)	+	+	+	+	+	+	+	+
Esculin (7 days) (browning[a]/fluorescence[b])	+w/−	+w/−	+w/−	+w/−	+w/−	+w/−	+w/−	+w/−
Hypoxanthine	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+
Urea (Christensen)	+	+	+	+	+	+	+	+
Xanthine	−	−	−	−	−	−	−	−
Utilization:								
Acetamide (7 days)	−	−	−	−	−	−	−	−
Citrate (7 days)	+	+	+w	+w	−	+w	+	+
Growth in lysozyme	+	+	+	+	+	+	+	+
Nitrate reduction (0.2 %)	+	+	+	+	+	+	+	+
Nitrite reduction (0.01 %)	−	−	−	−	−	−	−	−
Lysis of 5 % rabbit blood agar plate	+	+	+	+	+	+	+	+
Lysis of 5 % sheep blood agar plate	−	−	−	−	−	−	−	−

Table 1 continued

Characteristics	W9042	W9247	W9290	W9319	W9846	W9851	W9865	W9908
Resistance to[c]:								
Clarithromycin (≥8 μg/ml)	R	R	R	R	R	R	R	R
Ciprofloxacin (≥4 μg/ml)	R	R	R	R	R	R	R	R
Imipenem (≥16 μg/ml)	R	R	R	R	R	R	R	R
Minocycline (≥8 μg/ml)	I	I	I	I	I	I	I	I

All data from this investigation unless indicated otherwise

− negative, + positive, +w weak positive, *I* intermediately resistant, *R* resistant

[a] Browning following the protocol from Remel fact sheet

[b] Fluorescence according to Weyant et al. (1996)

[c] The MIC resistance breakpoints that were used are those of the CLSI (2011)

(Berd 1973). Colonies grown on TSA with 5 % sheep or HIA with 5 % rabbit blood were observed to be irregular, elevated, white after 3 days then turning light orange to tan with molar tooth shaped colonies after 7 days. Aerial and substrate hyphae were observed to be present with abundant true branched filaments and grossly visible aerial hyphae after 5 days. Hemolysis was observed on HIA with 5 % rabbit blood but not on TSA with 5 % sheep blood after 7 days at 35 °C. The isolates were found to grow well on both Middlebrook and Cohn 7H11 and heart infusion agars. Colonies were observed to be pale yellow on the bottom and pale orange on top on Middlebrook and Cohn 7H11 agar after 3 days. Small, abundant, white colonies were observed following growth on heart infusion agar after 3 days. As described by Goodfellow and Maldonado (2012), the morphologic characteristics for all eight clinical isolates are consistent with the members of the genus *Nocardia*.

Table 1 shows the results for the utilization of sole carbon sources and decomposition tests for the novel clinical isolates. Strain W9851^T was selected to represent the clinical isolates as the type strain. The phenotypic tests showed a wide range of phenotypically consistent characters that were able to clearly distinguish the clinical isolates from their closest phylogenetic neighbours such as the *N. altamirensis* and *N. brasiliensis* type strains (Table 2). Results for antimicrobial susceptibility testing showed that all the isolates were resistant to ciprofloxacin, clarithromycin and imipenem but were susceptible to amikacin, amoxicillin/clavulanate, linezolid; and seven of eight clinical isolates were susceptible to trimethoprim/sulfamethoxazole.

Chemotaxonomic characteristics

Whole-cell hydrolysates for the eight clinical isolates were found to contain *meso*-diaminopimelic acid as the sole whole cell-wall diamino acid with arabinose and galactose as the principle diagnostic whole-cell sugars (cell-wall chemotype IV sensu Lechevalier and Lechevalier 1970). Glucose, ribose and mannose were detected in minor quantities. The predominant menaquinone (average 61.6 %) was identified as MK-8-$(H_4)_{\omega\text{-cyc}}$; menaquinones MK-9 and MK-8-(H_2) were also detected averaging 19.2 and 10.5 %, respectively. Analysis of fatty acids indicated the presence of palmitic acid ($C_{16:0}$,39.4 %), tuberculostearic acid (10-methyl $C_{18:0}$, 16.2 %), oleic acid ($C_{18:1\ cis9}$, 15.5 %), and $C_{16:1\ t9}$, 15.4 %). Predominant phospholipids were found to include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. Mono- and di-unsaturated mycolic acids were detected in the range of 38–60 carbon atoms with peak amounts at 42 carbon atoms. Together the chemotaxonomic characters are consistent with other *Nocardia* species (Kämpfer et al. 2004).

Overall the phenotypic profiles obtained for the eight clinical isolates were clearly different from the profiles observed for the closest phylogenetically related *Nocardia* type strains allowing species-level identification (Table 2). MALDI-TOF mass spectra of the clinical isolates formed a coherent species level cluster within the genus *Nocardia* adjacent to the lineages of *N. brasiliensis* and *N. altamirensis* but distinctive from other species (Fig. 2).

Table 2 Phenotypic properties that distinguish the *N. vulneris* clinical isolates from the type strains of their closest phylogenetically related neighbors

Characteristics	Clinical isolates ($n = 8$)	*N. altamirensis* DSM 44997^T	*N. brasiliensis* ATCC 19296^T	*N. iowensis* DSM 45197^T	*N. tenerifensis* DSM 44704^T
Utilization of:					
Adonitol	−	+	+	+	−
L-arabinose	−	−	+	−	+
D-cellobiose	−7/8	+	−	−	−
Dulcitol	−	−	−	−	+
D-fructose	+	+	+	−	+
D-galactose	+	+	+	−	+
Glycerol	+	−	−	+	+
Lactose	−	−	+	−	−
Maltose	−	+	+	+	+
D-mannitol	+	+	+	−	+
Mannose	+	+	−	+	+
Melibiose	−	+	−	−	−
Raffinose	−6/8	−	−	−	−
Salicin	+	+	+	+	−
D-sorbitol	−	+	−	−	+
Sucrose	−	+	+	+	+
Trehalose	+	+	+	−	−
Growth at 35 °C	+	−	+	+w	+
Growth at 45 °C	−	−	−	+w	+w
Hydrolysis of:					
Adenine (21 days)	+	−	−	−	−
Casein (14 days)	+	−	+	+	−
Hypoxanthine	+	−	+	+	+
Tyrosine	+	−	+	+	−
Urea (Christensen)	+	+	−	+	+
Acetamide (7 days)	−	−	−	+	−
Nitrate reduction (0.2 %)	+	−	−	−	−
Lysis on 5 % rabbit blood agar	+	−	−	−	−
Antimicrobial resistance to[b]:					
Ampicillin (≥32 μg/ml)	S	S	R	R	R
Cefriaxone (≥64 μg/ml)	S	S	R	R	S
Clarithromycin (≥8 μg/ml)	R	I	R	R	I
Ciprofloxacin (≥4 μg/ml)	R	I	R	I	R
Imipenem (≥16 μg/ml)	R	S	R	I	S
Minocycline (≥8 μg/ml)	I	S	I	S	I

All data from this investigation unless indicated otherwise. Varaible results: number negative isolates/total number of isolates

− negative, + positive, +w weak positive, *I* intermediately resistant, *S* susceptible, *R* resistant

[a] Assimilation of carbon source/utilization with acid production

[b] The MIC resistance breakpoints that were used are those of the CLSI (2011)

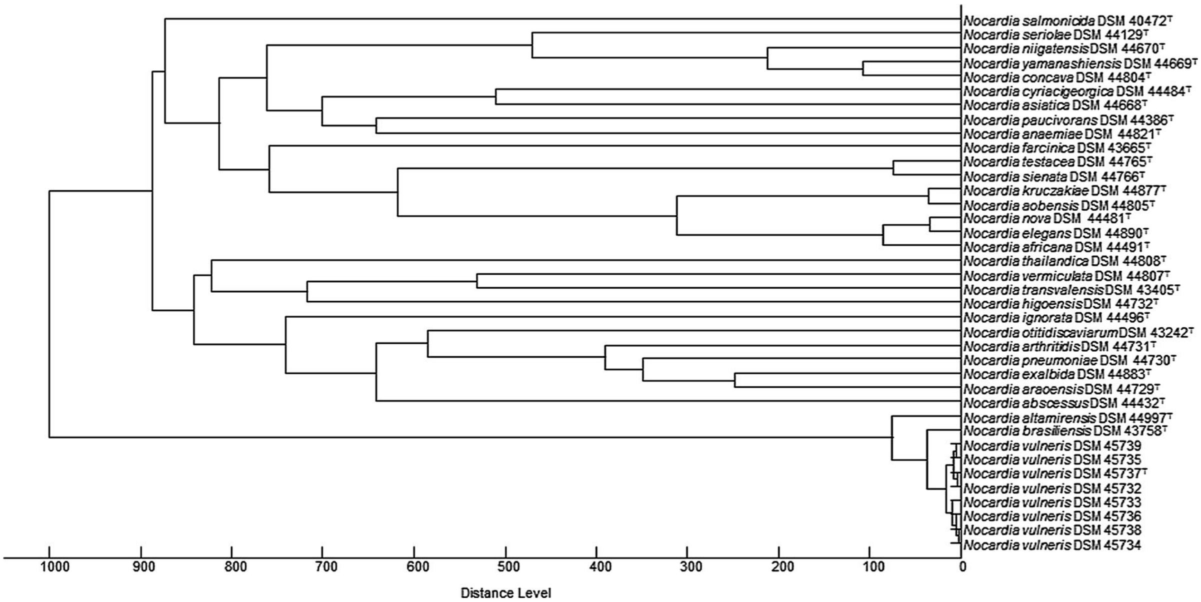

Fig. 2 Score-oriented dendogram generated by the MALDI-BioTyper software (version 3.1, Bruker Daltonics) showing the similarity of MALDI-TOF mass spectra of cell extracts of the clinical isolates and of selected type strains of the genus *Nocardia*. Mass spectra of the clinical isolates, of *N. brasiliensis* NBRC 14402^T and *N. altamirensis* DSM 44997^T were obtained in the present study. Spectra of all other type strains originate from the database of the MALDI Biotyper

Discussion

The morphologic and chemotaxonomic characters for the eight clinical isolates characterized in this investigation are consistent with classifying these isolates within the genus *Nocardia* (Goodfellow and Maldonado 2012). For instance, all strains produce aerial and substrate hyphae on TSA supplemented with sheep blood and pale orange to tan molar tooth shaped irregular colonies were observed. Microscopically, cells are cocco-bacilli with extensively branched hyphae. Analysis of bacterial protein composition by MALDI-TOF of the clinical isolates showed them to represent a distinct cluster within the genus *Nocardia.* The G+C ratio of 68.4 mol % is within the range previously observed for the genus *Nocardia*. Together, these results of the morphological and chemotaxonomic characters of the clinical isolates are consistent with those of the members of the genus *Nocardia.*

Analysis of the 16S rRNA gene sequences show the eight clinical isolates formed a distinct monophyletic clade within the genus *Nocardia* supported by a bootstrap value of 99 % and distinct from the *N. altamirensis* and *N. brasiliensis* type strains as shown in Fig. 1. Of importance are the measurements of DNA–DNA relatedness observed between clinical isolate W9851^T and *N. brasiliensis* NBRC 14402^T: laboratory and in silico DDH gave values of 72 and 66 %, respectively, whilst the ANI was 95.6 %. The laboratory generated DDH value falls within the transitional/borderline (or gray zone) around 70 % DNA–DNA relatedness as suggested by Wayne et al. (1987) indicating isolates within a species but lower than 80 % relatedness recommended as a boundary by Grimont (1988). The ANI value of 95.6 % is not conclusive, because it is exactly on the borderline for species delimitation (95–96 %) as recently described by Kim et al. (2014). However, the digital DDH value generated using GGDC 2.0 (Meier-Kolthoff et al. 2013a) delivered a resilient result with a confidence value (65.8 ± 2.9 % DDH) that supports the delimitation of the two strains. The 70 % DNA–DNA relatedness criteria suggested by Wayne et al. (1987) has been criticized by both Sneath (1989) and Felsenstein (2004) as indicative, but too rigid a boundary for species definition and for imposing an arbitrary division of species since inclusion within the theoretical limit does not ensure a monophyletic group. Values for DNA–DNA relatedness in laboratory experiments have been shown to be influenced by the potential for experimental

error (Sneath 1989), the experimental method, and disproportionate genome sizes, especially for values in the transitional gray zone, near the 70 % threshold. In such cases of significant but low DNA–DNA relatedness, phenotypic characteristics, and especially phenotypic coherence among isolates has been included as important considerations for species delineation (Vandamme et al. 1996; Wayne et al. 1987). Phenotypic characters should be unique and definable in order to clearly be recognized to provide for coherent classification. As shown in Table 2, the novel clinical isolates are readily distinguished phenotypically from the closest phylogenetically related *Nocardia* species; 11 of 33 phenotypic tests distinguish between W9851^T and *N. brasiliensis* NBRC 14402^T. Unlike *N. brasiliensis*, clinical isolate W9851^T is able to utilize glycerol and mannose but not adonitol, lactose or maltose. Compared to *N. brasiliensis* NBRC 14402^T, isolate W9851^T is able to hydrolyze adenine, utilize urea, reduce nitrate and able to lyse HIA rabbit blood after 7 days. Antimicrobial susceptibility profiles showed W9851^T to be susceptible to ampicillin and ceftriaxone, whereas the *N. brasiliensis* type strain is resistant. Wallace et al. (1988) reported on the use of common susceptibility profiles among strains to be an important adjunct method for species identification. In the present study, the eight clinical isolates were geographically limited to North America and only isolated from wounds, whereas *N. brasiliensis* has been associated with cutaneous infections and as the primary source of mycetomas in the Americas (Vera-Cabrera et al. 2013). The clinical isolates are differentiated from *N. altamirensis* by their ability to utilize glycerol, hydrolysis of adenine, casein, hypoxanthine and tyrosine, nitrate reduction and lysis of HIA supplemented with rabbit blood but are unable to utilize adonitol, D-cellobiose, maltose, melibiose, D-sorbitol, or sucrose. Phenotypic differences from *N. tenerifensis* include the inability to utilize salicin and trehalose, weak growth at 45 °C, ability to hydrolyze adenine, casein or tyrosine and nitrate reduction. *N. iowensis* is able to grow at 45 °C and use acetamide, but not able to hydrolyze adenine, utilize D-fructose, D-galactose, or D-mannitol, or lyse HIA supplemented with rabbit blood.

Analysis of near full length 16S rRNA gene sequences and MALDI-TOF profiles were able to clearly distinguish the new clinical isolates from other *Nocardia* species even though no definitive threshold limits have been suggested for analysis by MALDI-TOF. The results obtained in this investigation using polyphasic analysis are consistent with the clinical isolates being members of a novel species of the genus *Nocardia* for which *N. vulneris* sp. nov. is the proposed name.

Description of *N. vulneris* sp. nov. *N. vulneris* (vul'ne.ris. L. gen. n. *vulneris*, of a wound).

An aerobic, non-motile, Gram-stain positive, weakly acid-fast actinomycete obtained primarily from wound infections. Forms pale orange to tan, molar tooth shaped colonies with abundant aerial and substrate hyphae on HIA with rabbit blood, TSA with sheep blood, Middlebrook and Cohn 7H11 agar with OADC and heart infusion agar. Hemolysis of HIA supplemented with rabbit blood is observed after 7 days at 35 °C but not on TSA supplemented with sheep blood. Utilizes and produces acid from D-fructose, D-galactose, D-glucose, glycerol, i-myo-inositol, D-mannitol, mannose, salicin, and trehalose, but does not utilize adonitol, L-arabinose, D-cellobiose (most strains), dulcitol, i-erythritol, lactose, maltose, melibiose, raffinose (most strains), L-rhamnose, D-sorbitol, sucrose, and D-xylose. Utilizes citrate (most strains) as a sole carbon source but not acetamide as a carbon or nitrogen source. Grows in the presence of lysozyme, reduces nitrate but not nitrite, but has no arylsulfatase activity. Hydrolyses urea, adenine, casein, hypoxanthine, and tyrosine but does not hydrolyse xanthine. Esculin hydrolysis is weakly positive by browning but negative by UV light absorption. Grows at 25 and 35 °C but not 45 °C. Whole-cell hydrolysates contain *meso*-diaminopimelic acid and arabinose and galactose (cell-wall chemotype IV sensu Lechevalier and Lechevalier 1970). MK-8-$(H_4)_{\omega\text{-cyc}}$ and MK-9 are the predominant menaquinones with minor amounts of MK-8 (H_2). Polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The major fatty acids of the type strain are composed of palmitic acid ($C_{16:0}$), tuberculostearic acid (10-methyl $C_{18:0}$), oleic acid $C_{18:1\ cis9}$ and $C_{16:1\ t9}$. Stearic acid, pentadecylic acid and margaric acids are present in minor amounts. The principle mycolic acids are mono- and di-saturated and have a chain length of 38–60 carbon atoms. The DNA G+C

content of the type strain is 68.4 mol % (68.1 mol % from the 9.4 Mbp draft genome sequence).

The type strain W9851^T (= DSM 45737^T = CCUG 62683^T = NBRC 108936^T) was isolated from a 54-male patient with a leg wound in the state of Illinois. The GenBank accession number of the 16S rRNA gene sequence of the type strain is JN705252 and the accession number for the draft genome sequence is JNFP00000000.

Acknowledgments We thank Jean Euzéby for nomenclatural advice and Gabi Pötter (DSMZ) for help in chemotaxonomic analysis.

References

Baba T, Nishiuchi Y, Yano I (1997) Composition of mycolic acid molecular species as criterion in nocardial classification. Int J Syst Bacteriol 47:795–801

Berd D (1973) Laboratory identification of clinically important aerobic actinomycetes. Appl Microbiol 25:665–681

Cashion P, Hodler-Franklin MA, McCully J, Franklin M (1977) A rapid method for base ratio determination of bacterial DNA. Anal Biochem 81:461–466

Clinical and Laboratory Standards Institute (2011) Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standards M24-A. Clinical Laboratory Standards Institute, Wayne

Conville PS, Witebsky FG (2007) *Nocardia*, *Rhodococcus*, *Gordonia*, *Actinomadura*, *Streptomyces*, and other aerobic actinomycetes. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Phaller MA (eds) Manual of clinical microbiology, 9th edn. American Society for Microbiology, Washington, pp 515–542

Conville PS, Brown JM, Steigerwalt AG, Brown-Elliott BA, Witebsky FG (2008) *Nocardia wallacei* sp. nov., and *Nocardia blaklockiae* sp. nov., human pathogens and members of the "*Nocardia transvalensis* complex". J Clin Microbiol 46:1178–1184

Corti ME, Fioti MFV (2003) Nocardiosis: a review. Int J Infect Dis 4:243–250

De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 12:133–142

Felsenstein J (1981) Evolutionary trees from DNA sequences: maximum-likelihood approach. J Mol Evol 17:368–376

Felsenstein J (2004) Inferring Phylogenies, 1st edn. Sinauer Associates, Sutherland

Goodfellow M, Maldonado LA (2012) Genus I *Nocardia* Trevisan 1889. In: Goodfellow M, Kämpfer P, Busse H-J, Trujillo ME, Suzuki K-I, Ludwig W, Whitman WB (eds) Bergey's manual of systematic bacteriology, the *Actinobacteria*, 2nd edn. Springer, New York, pp 376–419

Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM (2007) DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol 57:81–91

Grimont PAD (1988) Use of DNA reassociation in bacterial classification. Can J Microbiol 34:541–546

Huss VAR, Festl H, Schleifer KH (1983) Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. Syst Appl Microbiol 4:184–192

Kämpfer P, Buczolitis S, Jäckel U, Grün-Wollny I, Busee H-J (2004) *Nocardia tenerifensis* sp. nov. Int J Syst Evol Microbiol 54:381–383

Kim M, Oh HS, Park SC, Chun J (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int J Syst Evol Microbiol 64:346–351

Klatte S, Kroppenstedt RM, Rainey FA (1994) *Rhodococcus opacus* sp. nov., an unusual nutritionally versatile *Rhodococcus* species. Syst Appl Microbiol 17:355–360

Kluge AG, Farris FS (1969) Quantitative phyletics and the evolution of anurans. Syst Zool 18:1–32

Kroppenstedt RM (1982) Separation of bacterial menaquinones by HPLC using reverse phase (RP18) and a silver loaded ion exchanger as stationary phases. J Liq Chromatogr 5:2359–2367

Kroppenstedt RM (1985) Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: Goodfellow M, Minnikin DE (eds) Chemical methods in bacterial systematics. Academic Press, London, pp 173–199

Lasker BA, Moser BD, Brown JM (2011) Gordonia. In: Liu D (ed) Molecular detection of human bacterial pathogens. CRC Taylor & Francis Group, Boca Raton, pp 95–110

Lechevalier MP, Lechevalier HA (1970) Chemical composition as a criterion in the classification of aerobic actinomycetes. Int J Syst Bacteriol 20:435–443

Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M (2013a) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics 14:60–73

Meier-Kolthoff JP, Göke M, Spröer C, Klenk H-P (2013b) When should a DDH experiment be mandatory in microbial taxonomy? Arch Microbiol 195:413–418

Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. Int J Syst Bacteriol 39:159–167

Minnikin DE, O'Donnell AG, Goodfellow G, Alderson G, Athalye M, Schaal A, Parlett JH (1984) An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. J Microbiol Methods 2:233–241

Rhuland LE, Work E, Denman RF, Hoare DS (1955) The behavior of the isomers of α, ε-diaminopimelic acid on paper chromatograms. J Am Chem Soc 77:4844–4846

Saitou N, Nei M (1987) The neighbor-joining method: a new method for constructing phylogenetic trees. Mol Biol Evol 4:406–425

Sasser M (1990) Identification of bacteria by gas chromatography of cellular fatty acids. USFCC Newsl 20:16

Sneath PHA (1989) Analysis and interpretation of sequence data for bacterial systematics: the view of a numerical taxonomist. System Appl Microbiol 12:15–31

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis

using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Töth EM, Schumann P, Borsodi AK, Kéki Z, Kovács AL, Márialigeti K (2008) *Wohlfahrtiimonas chitiniclastica* gen. nov., a new gammaproteobacterium isolated from *Wohlfahrtia magnifica* (Diptera: scarcophagidae). Intl J Syst Evol Microbiol 58:976–981

Trevisan V (1889) I Generi e le Specie Delle Batteriacee. Zanaboni and Gabuzzi, Milano

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbial Rev 60:407–438

Vera-Cabrera L, Ortiz-Lopez R, Elizondo-Gonzalez R, Ocampo-Candianu J (2013) Complete genome sequence analysis of *Nocardia brasiliensis* HUJEG-1 reveals a saprobic lifestyle and the genes needed for human pathogenesis. PLoS One 8:1–8

Wallace RJ Jr, Steele LC, Sumter G, Smith JM (1988) Antimicrobial susceptibility patterns of *Nocardia asteroides*. Antimicrob Agents Chemother 32:1776–1779

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandle O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Trüper HG (1987) Report of the *ad hoc* committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

Weyant RS, Moss CW, Weaver RE, Hollis DG, Jordan JG, Cook EC, Daneshvar MI (1996) Identification of unusual pathogenic Gram-negative and facultatively anaerobic bacteria, 2nd edn. Williams and Wilkins, Baltimore

Yamaguchi H, Sekimoto E, Shirakami A, Shibata H, Ozaki S, Shigekiyo T, Noda T, Shikji T, Kanda K, Hirose T, Matsuzawa T, Gonoi T (2013) Testicular nocardiosis accompanied by cutaneous lesions in an immunocompetent man. Intern Med 52:129–133

Yassin AF, Rainey FA, Brzezinka H, Burghardt J, Lee HJ, Schaal KP (1995) *Tsukamurella inchonensis* sp. nov. Int J Syst Bacteriol 45:522–527